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AUTOLOGOUS MARROW STEM CELL (MSC) TRANSPLANTATION  
FOR MYOCARDIAL REGENERATION

## TECHNICAL FIELD

The invention relates to autologous bone marrow  
stroma cells (MSCs) and the use of these cells in improving  
cardiac function in patients with heart failure. In  
particular, the present invention relates to the  
transplantation of MSCs into the myocardium to grow new  
muscle fibers. The specific aims of the invention include:  
1) using cell labeling techniques to confirm the survival  
and differentiation of implanted MSCs, and to identify  
their phenotype by both morphology and molecular markers;  
2) examining the effects of the micro-environment of the  
implanted MSCs, on their differentiation and phenotype  
expression; and 3) the functional contribution of MSCs when  
implanted into an ischemic segment of a myocardium.

## BACKGROUND ART

Heart failure is both common and deadly. In Canada,  
approximately 50,000 new cases of heart failure are  
diagnosed, and more than 300,000 patients currently suffer  
this condition. Heart failure is the only major  
cardiovascular disorder that is increasing in incidence and  
mortality at present, and in Class IV patients, one year  
mortality approaches 50% in spite of advances in drug  
therapy in recent years (L'enfant, C: Fixing the failing  
heart. Circulation 1997; 95:771-772).

Artificial heart and mechanical cardiac assist are  
largely used as a bridge to transplantation today, and they  
still face formidable difficulties of thromboembolic  
complications and suitable energy source. Cardiac  
transplantation can have dramatic improvements in terminal

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heart failure patients, but its availability is severely restricted by the donor availability, as well as by the complications associated with immunosuppression. These approaches are also very expensive, straining the limited health care resources.

One exciting new approach in recent years is tissue engineering, in which various cells are cultured *in vitro* over biodegradable polymer scaffolds to create a 3-dimensional construct *in vitro*, which can then be implanted to replace damaged tissues or organs. Upon the absorption of a biodegradable scaffold *in vivo*, these replacement tissues would not require immunosuppression if autologous donor cells were used for tissue engineering. Advances are being made in constructing cardiovascular structures, such as arteries and cardiac valves. Attempts are also being made to engineer 3-dimensional myocardial tissue blocks by seeding cardiac myocytes on a 3-dimensional scaffold, and cultured in rotating bioreactors. Without concomitant creation of a coronary vascular system within these constructs, however, such tissue engineered myocardium cannot be used therapeutically *in vivo*, as they will suffer rapid ischemic necrosis.

Such difficulties described above may be circumvented by tissue engineering neomyocardium *in vivo*. As is well known, cardiomyocyte loss from myocyte necrosis and apoptosis plays an important role in the initiation and progression of heart failure (Olivetti G, Abbi R, Quaini F, et al.: Apoptosis in the failing human heart. *N Engl J Med* 1997;336:1131-1141). Cellular cardiomyoplasty is a potential future therapy for heart failure in which donor cells with the potential to differentiate into cardiac myocytes are implanted into the damaged myocardium in order to regenerate new muscle fibers (Chiu RC-J, Zibaitis A, Kao

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RL: Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. Ann Thorac Surg 1995; 60:12-18). To date, a number of donor cells have been studied by various investigators, and are summarized below.

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#### A. Fetal Cardiomyocytes

Differentiated fetal cardiomyocytes retain a capacity for proliferation. Both in rodent and in canine models, fetal cardiomyocytes implanted into the myocardial wall of adult animals have been shown to be successfully engrafted, and develop into cells which are morphologically and functionally indistinguishable with the native cardiac myocytes within the recipient heart. They form gap junctions which should allow them to be depolarized and contract synchronously as a syncytium (Soonpaa MH, Koh GY, Klug MG, et al.: Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1994; 264: 98-101). By using fetal cells, however, the problem of donor cell availability becomes more formidable and the ethical issues more complex. Unlike for the use of fetal cells for treatment of neurological diseases, such as Parkinson's disease, in which engraftment of small numbers of cells may be adequate for therapeutic effect, fetal cardiomyocyte transplantation is likely to require millions of new cells to be efficacious, and continued proliferation of engrafted myocytes cannot be expected to expand the population, once they are removed from the donor embryos.

#### 30 B. Embryonic Stem Cells

Klug et al. transfected a transgene that confers resistance to a toxic drug into embryonic stem cells under the control of a cardiac specific promoter (Klug M.G.,

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Soonpaa M.H., Koh G.Y., Field L.J. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J. Clin. Invest. 1996; 98:216-24). When embryoid bodies derived from these stably  
5 transfected embryonic stem cells were exposed to the toxic antibodies, only cardiac myocytes survived. These cells were harvested and injected into the myocardial wall of the adult mice, where they engrafted and formed appropriate cell-to-cell junctions, i.e. intercalated discs with  
10 desmosomes and gap junctions, with host cardiomyocytes while maintaining a morphologically differentiated state. In principle, this strategy would allow for generating large numbers of donor myocytes. However, the ethical issues of using embryonic stem cells are currently hotly  
15 debated.

#### C. Modified Adult Cardiomyocytes and Myoblast Cell Lines

The adult cardiomyocytes are generally believed to be terminally differentiated and thus unable to  
20 proliferate. It has been previously reported that adult cardiomyocytes obtained from biopsy can be induced to proliferate *in vitro*, while retaining some phenotypic characteristics of the cardiac myocytes, and they can be successfully engrafted into the myocardium (Li RK, Jia ZQ,  
25 Weisel RD et al. Cardiomyocyte transplantation improves heart function. Ann. Thor. Surg. 1996; 62:654-660). It was shown that such cells implanted into an ischemic myocardium could improve ventricular function. These findings, however, require independent confirmation, more precise  
30 identification of cellular phenotype, and assurance against oncogenicity of the transformed cardiac myocytes before they can be considered for clinical use.

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Robinson et al. and other investigators implanted cells from established cell lines, such as C2 C12 cells which were originally derived from skeletal myoblasts (satellite cells) (Robinson SW, Cho PW, Levitsky HI et al.: Arterial delivery of genetically labeled skeletal myoblasts to the murine heart: Long-term survival and phenotypic modification of implanted myoblasts. Cell Transplantation 5:77-91, 1996). There is evidence that such cells, in spite of their origin from skeletal muscle, may transdifferentiate in the heart acquiring certain phenotypic characteristics of a cardiac myocyte, such as the expression of Connexin-43 and the formation of desmosomes at cell junctions. Although it is very convenient to use established cell lines which can be purchased from suppliers, the concern of oncogenicity upon transplantation *in vivo*, and the need for immunosuppression may limit their application in clinical therapy.

#### D. Adult Skeletal Myoblasts (Satellite Cells)

The feasibility of implanting autologous myoblasts (satellite cells) harvested from the adult skeletal muscle has also been explored (Chiu, RCJ, Zibaitis A, Kao L: Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. Ann. Thorac. Surg. 60:12-18, 1995). Using cell labeling techniques and phenotype specific antibodies, strong evidence has been presented that these myoblasts can undergo milieu-dependent transdifferentiation, and develop into striated muscle fibers with slow myosin heavy chains, as well as intercalated discs expressing Connexin-43. Taylor et al. confirmed that such engulfed satellite cells show ultrastructural features similar to immature cardiac myocytes, and when the implantation took place within a



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cryo-injured myocardium, cellular cardiomyoplasty could improve both the systolic and diastolic functions of such hearts (Taylor, D.A.; Atkins B.Z., Hungspreugs P., et al.: Regenerating functional myocardium: Improved performance  
5 after skeletal myoblast transplantation. Nat. Med. 1998; 4:929-933). Nevertheless, it would require sacrificing the patient's skeletal muscle, and the concern that the number of satellite cells in the skeletal muscle, as well as the satellite cells' mitotic potential may decrease with age.  
10 The optimal "conditions" for satellite cells to transdifferentiate into cardiomyocytes have not been clearly defined, and the molecular mechanisms of milieu-dependent differentiation remain unknown.

Thus, it would be highly desirable to identify a  
15 suitable cell type for use in effecting *in vivo* myocardial regeneration.

It would be further desirable to be provided with means to perform myocardial implantation without eliciting an immune response and without sacrificing the patient's  
20 skeletal muscle.

#### DISCLOSURE OF THE INVENTION

One aim of the present invention is to provide means to perform myocardial implantation without eliciting an  
25 immune response and without sacrificing the patient's skeletal muscle.

Another aim of the present invention is to use autologous MSCs in myocardial implantation to improve cardiac function.

30 Another aim of the present invention is to use autologous MSCs in myocardial implantation to effect the growth of new muscle fibers.

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Another aim of the present invention is to provide means for using a cell labeling technique to confirm the survival and differentiation of the implanted MSCs, and to identify MSCs phenotype by both morphology and molecular  
5 markers.

Another aim of the present invention is to provide means to examine the effects of the micro-environment of the implanted cells, on their differentiation and phenotype expression.

10 Another aim of the present invention is to provide means to examine the functional contribution of MSCs when they are implanted into an ischemic segment of the myocardium.

Another aim of the present invention is to provide  
15 a means for using autologous marrow stem cells for improving cardiac function, wherein said autologous marrow stem cells are introduced *in situ* into a myocardium.

The bone marrow stroma micro-environment is a complex network of cells and extracellular matrix, which  
20 maintains the hematopoietic system throughout the life of the individual. Hematopoietic cells are found in the spaces between the marrow stroma. The marrow stroma cells (MSCs) are functionally defined as capable of supporting hematopoiesis (Lichtman MD: The relationship of stromal  
25 cells to hemopoietic cells in marrow. In *Long Term Bone Marrow Culture*, pp. 57-96, DG Wright, JS Greenberger (eds), Alan R. Liss, New York, 1984), but lacking hematopoietic determinants (e.g., CD45, CD34, CD41, CD14, T- or B-cell markers and Mac-1).

30 Recent evidence showed that MSCs play a role in the "mesengenic process" for self-repair (Caplan AI: The mesengenic process. *Clinics Plast Surg* 1994;21:429-435). The body has developed two major strategies for tissue

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replacement and renewal. The first way the body attempts cell repair is predicated on the remaining proliferative capacity of differentiated, functioning cells, such as hepatocytes and endothelial cells. The second way is by their regeneration from residual cycling stem cells. An example in this category is the blood cells. All cells of the hematopoietic lineage are derived from a limited number of self-renewing multi-potent cells which respond to the appropriate cytokines and growth factors for differentiation.

It is now clear that bone marrow also contains cells that meet the criteria for stem cells of non-hematopoietic tissue. These cells are referred to as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchyma. These cells are also known as marrow stroma cells (MSCs), because they appear to arise from the supporting structures found in bone marrows.

The presence of stem cells for non-hematopoietic cells in bone marrow has been suggested. In addition, the possibility that bone marrow might be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair has been suggested, although not fully substantiated (Friedenstein A.J. et al. in Exper. Hematol. 1976; 4:276). According to the study of Freidenstien et al. samples of whole bone marrow were placed in plastic culture dishes and after about 4 hours, poured off the cells that were non-adherent to the dish, which in effect removed most of the hematopoietic stem cells and their progeny. After passage several times in culture, the adherent cells became more uniformly spindle-shaped in appearance. These cells illustrated the ability to differentiate into colonies that resembled small



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deposits of bone or cartilage. Further studies confirmed that MSCs isolated by the procedure of Friedenstein et al. were multi-potential, and readily differentiated into osteoblasts, chondroblasts, adipocytes, and myoblasts. It was further demonstrated that the MSCs, even after 20 to 30 cell doublings in culture were able to maintain their characteristics as multi-potential stem cells, capable of differentiating into various mesenchymal cells listed above.

10           Pereira R.F. et al. (Pereira R.F., Halford K., O'Hara M.D. et al.: Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc. Natl. Acad. Sci. 1995; 92:4857-4861) isolated MSCs using the technique of  
15   Friedenstein et al. from transgenic mice expressing a mutated collagen gene, which could be used as a cell marker by producing mutated Type I collagen molecules. These MSCs were injected intravenously into mice without this gene  
20   marker. After one week, few of the donor cells were found in the recipient mice. At one and five months, however, the donor cells accounted for 1.5 to 12% of the differentiated cells in bone, cartilage, and lung in addition to marrow and spleen. It appeared that the donor MSCs first replaced  
25   a portion of the MSCs in the bone marrow of the recipient mice. The MSCs then participated in a normal biological cycle in which MSCs in the bone marrow served as a continuing source of progenitor cells for a variety of mesenchymal tissues in the body. These findings suggested that the progeny of MSCs acquired the phenotype of  
30   different target tissues, either before they left the marrow, or after they have entered the micro-environment of the tissue itself through "milieu-dependent differentiation". Thus, it was postulated that MSCs

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participate in the "mesengenic process" which continues throughout life (Caplan A.I.: The mesengenic process. Clinics Plast. Surg. 1994; 21:429-435). This process functions to continually rejuvenate various mesenchymal tissues and ensure rapid repair of tissue injuries. Such repair involves the recapitulation of the same cellular transitional events observed during embryonic development on the scaffolding of the pre-existing macro-morphology and its signaling systems. Clinical applications of these findings have been attempted (Horwitz EM, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nature Medicine 5, 309-313, 1999).

MSCs have been shown to differentiate to cardiac myocytes *in vitro* under proper conditions (Makino S. Fukuda K, Miyoshi S, et al.: Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J. Clin. Invest. 1999; 103:697-705). After immortalizing MSCs by prolonged culture *in vitro*, they were able to identify a single clone of adherent fibroblast like cells which, when treated with 5-azacytidine, would reproducibly differentiate into adjoining myocytes with synchronous beating. Analysis of the isoforms of contractile protein genes, such as myosin heavy chain, myosin light chain,  $\beta$ -actin, indicated that the phenotype of these cells was similar to that of fetal ventricular cardiomyocytes. After differentiation, these cells, which they called cardiomyogenic cells, acquired many morphologic features of cardiac muscle, including sarcomeres, one to three centrally located nuclei, and atrial granules. They also expressed several cardiac specific genes, including the GATA4 and Nkx2.5 transcription factors and the brain natriuretic peptide (BNP) as well as atrial natriuretic factor (ANF) genes.

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They stained positive with anti-myosin, anti-desmin, and anti-actinin antibodies. In addition, they displayed cardiac like action potentials with a shallow resting membrane potential, long action potential duration, as well as a late diastolic slow depolarization current.

However, it should be emphasized that these findings were obtained and observed *in vitro* only, and no *in vivo* studies had been reported before the present invention.

The myocardium belongs to mesenchymal tissues. In accordance with an embodiment of the present invention, the myocardium was investigated as a target recipient of MSCs for effecting *in vivo* MSCs differentiation for improving cardiac function.

In accordance with an aspect of the present invention, bone marrow stoma cells (MSCs) were employed in *in vivo* myocardium implantation and found to effect growth of new muscle fibers and improve overall cardiac function.

In accordance with the present invention, there is provided a use of autologous MSCs for improving cardiac function wherein said MSCs are transplanted *in situ* into a myocardium.

In accordance with another aspect of the present invention, there is provided a use of autologous MSCs for improving cardiac function wherein said MSCs are transplanted *in situ* into a myocardium, and differentiate into cardiomyocytes, fibroblasts and endothelial cells.

In accordance with the present invention, there is provided a method of improving cardiac function in a patient with heart failure without eliciting an immune response and without sacrificing the patient's skeletal muscle; which comprises the step of transplanting autologous bone marrow stroma cells (MSCs) into said patient's myocardium to grow new muscle fibers.

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The method may further comprise the step of using a cell labeling technique to confirm survival and differentiation of implanted MSCs, and to identify said MSCs phenotype by both morphology and molecular markers.

5           The method may further comprise examining the effects of the micro-environment of implanted MSCs on their differentiation and phenotype expression.

10           The method may further comprise examining functional contribution of MSCs implanted into an ischemic segment of the myocardium.

The transplanting may be effected in the myocardium *in situ*, in the myocardium artery or using a catheter from within the myocardium.

15           The transplanting may also be effected in association with angiogenesis factors.

In accordance with a further aspect of the present invention, there is provided a method of treating cardiac failure, said method comprising (a) retrieving bone marrow from a patient suffering from cardiac failure;  
20           (b) isolating marrow stroma cells from said bone marrow;  
             (c) expanding said marrow stroma cells in culture; and  
             (d) introducing said marrow stroma cells into a myocardium of said patient.

25           In accordance with a yet further aspect of the present invention, there is provided a use of autologous marrow stroma cells for examining the effects of a myocardial micro-environment on marrow stroma cell differentiation, wherein said autologous marrow stem cells are introduced *in situ* into an ischemic segment of a  
30           myocardium of an animal model.

Recently, preliminary studies were carried out using autologous bone marrow stroma cells (MSCs) as donor cells for myocardial implantation.

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In accordance with the present invention, the rationale for using MSCs as donor cells for cellular cardiomyoplasty will be evident.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates four days after implantation of MSC into the myocardium; Top panel: Hematoxylin and eosin stain; Lower panel: Fluorescent microscopy picture, showing MSCs labeled with DAPI;

10 Fig. 2 illustrates four weeks post-implantation;

Fig. 3 illustrates specimen four weeks after MSC implantation into the myocardium; Top panel: Hematoxylin and eosin stain; Lower panel: Fluorescent cells originated from MSCs labeled with DAPI *in vitro*;

15 Fig. 4 illustrates rat MSCs morphology in culture, and in particular, a phase contrast photomicrograph of twice-passaged culture of MSCs just before implantation. Most adherent MSCs are practically fibroblastic in morphology. Scale bar represents 30 $\mu$ m;

20 Fig. 5 illustrates histochemical staining for  $\beta$ -galactosidase activity of rat MSCs in culture. The transfected MSCs showed clear staining for  $\beta$ -galactosidase activity. Transfection efficiency of the MSCs was approximately 100%. Scale bar represents 60 $\mu$ m;

25 Fig. 6 illustrates  $\beta$ -gal positive cells trapped within a coronary capillary immediately after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by H & E stain. Arrow, a capillary endothelial cell. Scale bar represents 15 $\mu$ m;

30 Fig. 7 illustrates  $\beta$ -gal positive cells with i morphology outside infarct scar 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed



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by H & E stain. Arrows, intercalated disk-like structure. Arrowhead, the nucleus of a  $\beta$ -gal positive cell. Scale bar represents 15 $\mu$ m;

Fig. 8 illustrates  $\beta$ -gal positive cells with fibroblast-like morphology in the myocardial scar 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by H & E stain. Arrows,  $\beta$ -gal positive cells. Scale bar represents 1.5mm. (Inset) Higher magnification of the area in square. Scale bar represents 30 $\mu$ m;

Fig. 9 illustrates  $\beta$ -gal positive cells incorporated into endocardium 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by Eosin stain. Arrow, endocardium. Scale bar represents 15 $\mu$ m;

Figs. 10A & 10B illustrate  $\beta$ -gal positive cells incorporated into coronary capillaries 4 weeks after MSCs injection. Staining for  $\beta$ -galactosidase activity followed by H & E stain. A. Outside the infarct scar. Arrow, a capillary with  $\beta$ -gal positive cells in the subendocardial fibrosis area. Arrowhead, normal myocardium. Asterisk, endoventricular space. Scale bar represents 600 $\mu$ m. (Inset) Higher magnification. Arrow, the cross section of the same capillary with  $\beta$ -gal positive cells. Scale bar represents 15 $\mu$ m. B. In the infarct scar. Arrow, the oblique section of a capillary with  $\beta$ -gal positive cells. Scale bar represents 15 $\mu$ m; and

Fig. 11 illustrates histochemical stain for connexin-43 in the intercalated discs (arrows), demonstrating the presence of gap junction unique to myocardium in the labeled (blue) myocytes.

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## BEST MODE(S) FOR CARRYING OUT THE INVENTION

In accordance with the present invention, there is provided means to perform myocardial implantation without eliciting an immune response and without sacrificing the patient's skeletal muscle.

The ability to regenerate a functioning cardiac muscle in patients with heart failure, who have lost a significant amount of native cardiac muscle fibers through ischemic necrosis and apoptosis, has enormous therapeutic potential in the treatment of heart failure. Autologous marrow stroma cells (MSCs) have been identified as target donor cells for eliciting myocardial regeneration in vivo. Using autologous MSCs as donor cells for cell transplant therapy has a number of important advantages. In particular, by using autologous MSCs in cell transplant therapy, the need for fetal tissue and its ethical and legal controversies can be avoided, while also avoiding the need for immunosuppression. Unlike using modified cardiac myocytes or established cell line myoblasts, the danger of oncogenicity can be diminished. In addition, using autologous skeletal myoblasts would require the sacrifice of a patient's skeletal muscle, which is irreplaceable. In contrast, bone marrow puncture, a routine clinical procedure, can be repeated to harvest MSCs for more than one occasion. The procedure is also much less invasive, and can be easily performed in patients under local anesthesia, in contrast to the excision of a major muscle mass, such as the latissimus dorsi muscle in order to harvest satellite cells. The latter procedure would likely require general anesthesia. Considering that such patients will be already suffering from severe heart failure, invasiveness of the procedure required for donor cell harvesting could be an important clinical consideration. The cell implantation

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procedure can be combined with other surgical operations, such as coronary bypass surgery, or by minimally invasive surgical techniques or by transvenous catheter injections.

Possible problems include false positive results associated with some cell labeling techniques as well as the interpretation of phenotype specificity associated with immunohistochemical findings. For example, a myosin-slow molecule may be detected both in Type I skeletal muscle fibers as well as in cardiac myocytes, and Connexin 43 may be expressed in immature myoblasts. Nevertheless, by employing several different cell labeling techniques and immunostain antibodies, correlating with histological and ultrastructural examinations, much of these uncertainties may be addressed.

Previous proposals to use certain sources of donor cells for cellular cardiomyoplasty are not ideal for clinical application, owing primarily to the need for fetal tissue, and/or to the need for immunosuppression (Soonpaa M.H., Koh G.Y., Klug M.G., Field L.J. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1994;264:98-101; Li R.K., Jia Z.Q., Weisel R.D., Mickle D.A.G., Zhang J., Mohabeer M.K., et al. Cardiomyocytes transplantation improves heart function. Ann Thorac Surg 1996;62:654-61; Taylor D.A., Atkins B.Z., Hungspreugs P., Jones T.R., Reedy M.C., Hutcheson K.A., et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. Nature Medicine 1998;4:929-33; Klug M.G., Soonpaa M.H., Koh G.Y., Field L.J. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J. Clin. Invest. 1996; 98:216-24).

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The present invention demonstrates that the *in vivo* myocardial environment can support the growth and induce the cardiomyogenic differentiation of MSCs. Compared to other cell sources, the present invention illustrates the advantages in the clinical use of MSCs for cellular cardiomyoplasty.

Grafting of cells into the myocardium requires some form of delivery system. The choice for the routes of cell implantation may depend on the pathology of the heart. Up to now, most of studies in the field of cellular cardiomyoplasty were performed by direct injection of various cells into the myocardium. Though implanted cells may have the ability of migration along the ventricular surface of heart (Connold A.L., Frischknecht R, Dimitrakos M, Vrbova G. The survival of embryonic cardiomyocytes transplanted into damaged host rat myocardium. J Muscle Res Cell Motil 1997;18:63-70), this procedure covers only a limited field and may require multiple injections, either through epicardium, or via the endocardium. Coronary arterial delivery of donor cells to the myocardium possesses theoretical advantages, at least for certain types of heart failures. Thus, in order to optimize the strategy for cell implantation, the feasibility of delivering MDSCs by selective infusion into the coronary circulation was evaluated.

In accordance with the present invention MSCs are infused into coronary artery and appear to repopulate the heart. Further, signals originating in the cardiac milieu appear to modify the developmental program of the infused MSCs. Studies conducted in accordance with the present invention further confirm the residence of MSCs outside of the capillary bed, and illustrate the structural

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interactions between the host myocardial tissues and the implanted MSCs.

To trace the fate of infused MSC according to an embodiment of the present invention, MSCs were retrovirally transfected with  $\beta$ -gal reporter gene for cell labeling. Compared with other virus-based gene transfer, retrovirus has less immunological response and longer gene expression (Onifer SM, White LA, Whittemore SR, Holets VR. *In vitro* labeling strategies for identifying primary neural tissue and a neuronal cell line after transplantation in the CNS. *Cell Transplantation* 1993;2:131-149). Using current transfection model, the transfection efficiency in culture is approximately 100% without obvious adverse effect on the cell growth. Our *in vivo* control studies, including infusion of MSCs culture supernatant, non-transfected MSCs or lysed transfected MSCs while performing the same read-out, confirmed the specificity of this cell labeling technique.

Immediately after the infusion, the MSCs were trapped within the coronary capillaries in the non-infarct area. The reason MSCs could not be found in the infarct scar at this time may be related to the complete occlusion of involved coronary artery (Left coronary artery). However, 4 weeks after infusion, the MSCs could be found both in the infarcted scar and in non-infarct area outside the vascular structure. The mechanism of the translocation of MSCs from the vascular lumen into the myocardial interstitium is unknown. MSCs may migrate out of the vasculature and move from the non-infarct area to the infarction scar. The other possible explanation is that scar of myocardial infarction is not a completely dead tissue. The reason why MSCs can only be found in 60% of rats (6 out of 10) 4 weeks after injection may be related



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to the infusion technique. By using this model of briefly clamping the ascending aorta distally, some cells may have leaked out of the puncture hole on the ascending aorta. Others may escape through the blood stream to distal organs and tissues.

MSCs in different myocardial microenvironments clearly have different fates. In the non-infarct area, they express the phenotypes of normal cardiomyocytes and connected with surrounding host cardiomyocytes by intercalated disk-like structure. In the infarct scar, they appear primarily fibroblast-like. This homing ability and the capability to acquire the phenotypes of different target tissues suggest that the microenvironment plays a significant role for the differentiation of these cells.

According to the present invention, in contrast to myocardial scar tissue, the normal myocardial microenvironment appears to enable newly arrived cells to be exposed, in an appropriate sequential manner, to various cardiomyogenic specific growth factors and differentiation molecules, such that the infused MSCs could develop into fully mature cardiomyocytes. The fibroblast-like MSCs seen in the infarction scar could have differentiated into primary fibroblast, which are mature mesenchymal cells or they could still maintain the multipotent differentiation ability for future maturation.

The studies of the present invention revealed that some  $\beta$ -gal positive donor cells differentiate into endothelium, which was incorporated into capillaries in the infarct and non-infarct areas. Accordingly, these marrow-derived endothelial progenitor cells are likely to be involved in the angiogenesis and vasculogenesis in the remodeling process of myocardial infarction. Thus further

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suggesting the potential of use of MSCs for implantation into the myocardium to improve heart function.

In addition, it has been shown that some cells in MSCs culture are positive for Factor VIII-associated antigen (Singer JW, Charbond P, Keating A, Nemunaitis J, Raugi G, Wight TN, et al. Simian virus-40 transformed adherent cells from human long-term marrow cultures: Clone cells produced with "stromal" and hematopoietic characteristics. Blood 1987;70:464-474), suggesting an endothelial origin. Shi et.al suggested that a subset of cells localized in the bone marrow could be mobilized to the peripheral circulation and colonize endothelial flow surfaces of vascular prostheses (Shi BQ, Rafii S, Wu MHD, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362-367).

Both localized site-specific and global delivery of autologous MSCs may be of potential therapeutic benefit in view of different cardiac pathology. For example, intracoronary delivery of MSCs may be more suitable for the treatment of heart failure due to diffuse cardiomyopathy. Furthermore, the present invention demonstrates that when expanded marrow-derived stromal cells are delivered to the coronary circulation of an infarcted heart, they are capable of populating the heart and differentiating along several lineages including cardiomyocytes, fibroblast and endothelial cells.

The findings of the present invention suggest that infarcted heart muscle can signal mobilization of MSCs to enter circulation, and reach the coronary artery, where they may participate in myocyte replenishment, reactive fibrosis and scar formation as well as angiogenesis in the post-infarct pathophysiological remodeling process,

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involving both the infarcted segment and the remote non-infarcted areas. Additional studies in accordance with the present invention will further elucidate the role of MSCs in myocardial infarction and the clinical applications of MSCs implantation. It is fully contemplated that the findings presented in accordance with the present invention will enable therapeutic modulation of the remodeling process after myocardial infarction, in both animals and humans.

10

#### Example I

#### Preliminary Studies

In our preliminary study, isogenic rats were used as donors and recipients, since as in the autotransplants, this model obviates the need for immunosuppression. Lewis rats weighting 175 to 200 grams were used in all experiments. As will be described in detail below, femoral and tibial bones were explanted from the donor rats and used as the source for bone marrow stroma cells. Details of isolation, plating and passaging techniques for MSC will also be described below. The MSCs were expanded in culture for 2 to 3 weeks and labeled with DAPI (4', 6-diamidino-2-phenylindole) before transplanting them into the lateral wall of the left ventricle of the recipient rat hearts. This was accomplished by direct injection of MSC suspension using a 28-gauge needle. At different time intervals ranging from 2 days to 4 weeks following the implantation, the hearts were harvested and studied histologically. The hearts at the implant sites were sectioned serially, and stained with hematoxylin and eosin. Slides of the adjacent section to the above were examined under fluorescent microscopy to identify DAPI, which upon binding to the DNA

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of the MSCs prior to implantation, can be recognized as fluorescent positive cells.

The sections (6  $\mu$ m/thick) were also examined immunohistochemically using antibodies against myosin-slow molecules. Other serial sections from these specimens are currently undergoing studies using antibodies against the cardiac gap junctional protein, Connexin-43, and other phenotype specific antibodies, for immunolabeling and staining.

Fig. 1 illustrates a microphotograph obtained 4 days after implantation. The implant site shows a needle track created during the process of injecting the MSCs, with some inflammatory response and fibrosis within the needle track. Fluorescent microscopic examination demonstrated the presence of the labeled MSCs implanted. Fig. 2 shows labeled cells in the injection site, immunolabeled with myosin-slow antibodies which stains red; DAPI labeled fluorescent cells within needle track made during implantation; and immunohistochemical stain with antibody against slow myosin heavy chain, which shows red color. The triangle marker points to the native cardiac myocyte, and arrows point to the appearance of myosin molecules in the cytoplasm of implanted cells.

The deep red color shows myosin-slow heavy chains in the native muscle (triangle), whereas the red stain adjacent to the labeled cells suggest the synthesis of myosin-slow molecules in the implanted cells (arrows). Fig. 3 is a photograph taken a short distance away from the implant needle track, showing migrated or infiltrated MSCs appearing to have differentiated fully and were incorporated into cardiac muscle fibers, morphologically indistinguishable with the native myocardium. Clear labeling of these cells can be demonstrated under

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fluorescent microscopy. Morphologically, they appear identical to the native myocardial fibers. Photographs were taken from the myocardium adjacent to the needle track, where the implanted cells had migrated or infiltrated.

5 Additional studies are described below to confirm these findings, as well as to further elucidate the phenotypes of the new muscle using additional specific antibodies. These preliminary studies, however, clearly demonstrated various experimental techniques, ranging from  
10 isolation, culture and identification of MSCs, as well as implanting of these cells to the rat hearts with virtually no mortality.

#### 15 Experimental Protocol: The Rationale for Experimental Models

In clinical application of the present invention, use of autologous MSCs for cardiac implantation to improve cardiac function is particularly advantageous in that it avoids the need for immunosuppression. Relatively  
20 inexpensive isogenic Lewis rats were chosen for the preliminary study. The preliminary investigation described above indicates that this is a useful and reliable model, with little operative mortality for cardiac cell implantation resulting. Furthermore, the capability to  
25 perform coronary artery ligation and sequential echocardiographic studies in such animals is confirmed and the feasibility of proposed experiments in accordance with the present invention is supported.

#### 30 Experimental Design

For each rat receiving cardiac cell implant (experimental animal), there will be an isogenic rat to serve as the donor of bone marrow stroma cells. Another group of sham operated rats (controls) will undergo



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identical surgical procedures as the experimental animals, but will receive injection of cell culture media without MSCs.

5 a) Donor rats will be sacrificed, and their femoral and tibial bones will be used to isolate, select and culture MSCs *in vitro* for 2 weeks using the technique described below. Then the cells will be collected, labeled and injected into the myocardium of the experimental recipient rats.

10 b) Cell implant recipient (experimental) rats: The prospective future recipients for cell implant will undergo thoracotomy and ligation of the anterior descending coronary artery (see below). The chest will be closed and the animals monitored with weekly echocardiographic studies  
15 to observe changes in the ventricular wall motion of the ischemic zone for 2 weeks. In the second thoracotomy, these rats will receive injection of isogenic MSCs cultured and labeled *in vitro*. The injections will be made through a 26-gauge needle into the anterolateral wall of the left  
20 ventricle, both at the center of the infarct zone, as well as at the peripheral border zone between the infarcted and non-infarcted cardiac muscles. Following the implant procedure, the chest will be closed and studied weekly using echocardiography as described below. They will be  
25 sacrificed after cell implantation at an interval of 4 days, 2 weeks, 4 weeks, and 3 months, with a sample size of 10 rats each. Frozen sections will be made serially through the implant site, at a thickness of approximately 6  $\mu$ m each, and slides will be process for fluorescent microscopic,  
30 histological, immunohistochemical and electron microscopic studies as described in detail below.

c) Sham operated (control) rats: This group will undergo coronary artery ligation and cardiac implantation

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procedures exactly as described above for the experimental group. However, instead of receiving cultured and labeled MSCs, they will receive the same volume of culture media only. They will be harvested at the same time intervals and  
5 studied in the same manner as described for the experimental animals.

#### Sample Size

The sample size of 10 was based on our preliminary  
10 study as this is a highly reliable model that resulted in minimal operative mortality. In the future, wall motion studies will be performed to calculate the sample size required.

All animals will receive humane care and all  
15 experiments will be performed according to the "Guidelines to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

#### Isolation, Plating and Passaging Techniques of Bone 20 Marrow Stroma Cells

Isolation and primary culture of MSCs will be performed according to Caplan's method. After overdose with pentobarbital (100 mg/kg given intraperitoneally) (MTC Pharmaceuticals, Cambridge, Ontario), the femoral and  
25 tibial bones of the donor Lewis rats (weighing 175 to 200 grams) will be collected and the adherent soft tissue removed. Meticulous dissection of the long bones will be carried out in order to remove soft tissue to ensure that myogenic precursors are not carried into the bone marrow  
30 preparation. Both ends of the bones will be cut away from the diaphysis with bone scissors. The bone marrow plugs will be hydrostatically expelled from the bones by insertion of 18-gauge needles fastened to 10 ml syringes filled with complete medium; the needles are inserted into

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the distal ends of the femoral and proximal ends of the tibial bones, and the marrow plugs expelled from the opposite ends. The marrow plugs are disaggregated by sequential passage through 18-gauge, 20-gauge and 22-gauge needles and these dispersed cells are centrifuged and resuspended twice in complete medium. Cell viability is assessed by the trypan blue exclusion test. After the cells are counted in a hemocytometer,  $5 \times 10^7$  cells in 7-10 ml of complete medium are to be introduced into 60 mm polystyrene tissue culture dishes (Corning, Inc., Corning, NY), which are coated in advance with a layer of laminin (Sigma) to promote marrow stroma cell adherence. Three days later, the medium is changed and the non-adherent cells discarded. The medium is completely replaced every 3 days. In approximately 10 days after seeding, the dishes will become nearly confluent and the adherent cells can be released from the dishes with 0.25% trypsin in 1 mmol/L sodium ethylenediaminetetraacetic acid (Gibco Laboratories, Grand Island, NY), split 1:2, and seeded onto fresh plates. After these twice passaged cells become nearly confluent, they can be harvested and used for implantation experiments described below after being labeled with DAPI. The "complete medium" mentioned above for our culture consists of Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories) containing selected lots of 10% fetal calf serum (FCS; JR Scientific Inc., Woodland, CA), and antibiotics (Gibco Laboratories; penicillin G, 100 U/ml; streptomycin 100  $\mu$ g/ml, amphotericin B 0.25  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

30

#### Bone Marrow Stroma Cell Labeling

Although in our preliminary studies, we have used DAPI for cell labeling, retroviral vectors as the tool for

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cell labeling may also be employed. Likewise, other known techniques for cell labeling may be employed in accordance with the present invention. Retroviral vectors permit insertion of foreign synthetic genetic information in target cells. Reporter genes such as  $\beta$ -galactosidase, can therefore be stably integrated in chromosomal DNA. Expression of these transgenes permits ambiguous identification, by classic histochemical or fluorescence microscopy, of gene modified cells. As an example, retroviral labeling of hematopoietic stem cells is routinely carried out in the study of bone marrow transplantation in rodents. Undifferentiated (gene labeled) progeny cells of a widely different phenotype (lymphocytes, granulocytes, platelets, red blood cells) are readily detectable up to one year after bone marrow transplantation. We propose that retroviral labeled stroma cells that survive, proliferate or differentiate following cardiac implantation, will preserve and express the transgene as is classically observed in animals transplanted with retrovirus labeled hematopoietic stem cells. With this technique, important questions regarding bio-distribution of transplanted stroma cells can be addressed. Unambiguous identification of "labeled stroma cells", as well as their differentiated progeny, local and distant to the site of implantation in the injured heart, will become possible. Identification of other "unexpected" progeny cells, such as endothelial cells, interstitial cells, possibly others, will also be feasible. Furthermore, in accordance with an embodiment of the present invention sensitive PCR-based techniques were employed to detect as little as 1 to 100,000 transgene positive cells from tissue DNA extracts.

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According to an embodiment of the present invention cultured primary marrow stroma cells were readily transduced with synthetic retroviral vectors. In addition, high efficiency gene transfer in cultured stroma cells may also be employed and genetically labeled cells can be expanded *in vitro* for up to 3 to 4 weeks without loss of reporter gene expression (in this case the green fluorescent protein). We have on hand a high titer VSV-G pseudotyped  $\beta$ -gal retroviral producer. We propose that transplanted stroma cells genetically labeled with a  $\beta$ -galactosidase retrovector will be readily identified by classic X-Gal staining of cardiac tissue sections.

#### Coronary Artery Ligation Model in Rats

The recipient rats, both experimental and control, are isogenic Lewis rats weighing 175 to 200 grams. Anesthesia is induced and maintained with isoflurane (MTC Pharmaceuticals). The animals are intubated with an 18-gauge intravenous catheter and connected to a Harvard rodent ventilator (Harvard Apparatus Co., Inc., South Natick, Mass.) at 85 breaths per minute. The heart is exposed via a 1.5 cm left thoracotomy incision. Under direct vision, using a 5-0 prolene suture, the anterior descending coronary artery which is visible in the epicardium is ligated proximally. The thoracotomy is closed with 4-0 monofilament sutures. The muscle and skin layers are closed with 4-0 absorbable sutures and the animals are returned to their cages with filter tops. After the learning curve, the operative mortality is virtually nil.

#### Cell Transplantation into the Rat Heart

The recipient rats which underwent coronary artery ligation 2 weeks previously will undergo a second



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operation. Anesthesia and thoracotomy will be performed in the manner described above. Under direct vision, the MSC suspension is injected into the lateral wall of the left ventricle with a 20-gauge needle, both at the center of the ischemic segment of the myocardium, and at the border zone at the junction between the infarcted and normal myocardium. The thoracotomy closure and post-operative care are similar to that described above, and the animals sacrificed at intervals after this procedure, as stated earlier.

The sham operated control rats will undergo an identical procedure as described for cell implantation in the experimental animals. The only difference is that instead of injecting cultured MSCs, an identical volume of culture media (component described above) will be injected.

#### Echocardiographic Studies on Wall Motion

Transthoracic Doppler echocardiographic studies will be performed in the rats every week following implantation. The rats are anesthetized as described above, the chest wall shaved, and echocardiography performed using our echo system equipped with a 7.5 MHZ transducer (Hewlett Packard Sonos 2500). A 2-dimensional short axis view of the left ventricle is obtained at the level of the papillary muscle to record M-mode tracing. Anterior and posterior end-diastolic and end-systolic wall thickness and LV diameters are measured using the American Society of Echocardiography Lineage Method, from at least three consecutive cardiac cycles. The changes in wall thickness and ventricular segmental wall motion and diameter will be recorded on videotape, and assessed by blinded independent echocardiographers.

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## Morphological and Immunohistochemical Studies

The heart specimens obtained from the recipient rats at various intervals will be perfused with 100 ml of saline through the posterior wall of the left ventricle, avoiding the transplant area, then processed for frozen sections. The lateral wall of the left ventricle is isolated from the remainder of the heart. Sections 6  $\mu$ m thick are cut from the hearts and successive sections collected by gelatine coated glass slides. This ensures that different stains could be applied on successive sections of the tissue cut through the transplanted area (Figs. 1 and 3). One of the sections is mounted and stained with X-Gal, to identify and view the  $\beta$ -gal labeled donor cells. An adjacent section is stained with hematoxylin and eosin as described in the manufacturer's specification (Sigma Diagnostics) to depict nuclei, cytoplasm and connective tissue. Other adjacent sections will be immunolabeled using various antibodies for immunohistochemical evaluation in order to identify phenotypic expression at the molecular level. These antibodies include those against myosin-slow molecules, cardiac gap junctional protein Connexin 43, desmin, and sarcomeric myosin (MF 20). Finally, specimens will also be processed and sent for ultrastructural examination in our future studies.

## Example II

Bone marrow stromal cells (MSCs) were infused into ascending aorta of isogenic recipient rats after coronary artery ligation. MSCs were found to traffic through the coronary circulation to the injured heart, and form cardiomyocytes, fibroblasts and endothelial cells etc., depending on the specific microenvironment. Thus, autologous MSCs appear to participate in the post-infarct

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repair and remodeling process, and provide a therapeutically utility in treating heart failure and in improving cardiac function.

## 5 Methods

### Animals

Male inbred Lewis rats 200 to 250 gm were obtained from Charles River Laboratories. These isogenic rats were used as donors and recipients to simulate the autologous  
10 infusion of MSCs in the future clinical application. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press,  
15 revised 1996 and the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

### Myocardial Infarction Model

12 recipient rats were anesthetized with isoflurane  
20 (MTC Pharmaceuticals). Rats were intubated and ventilated at 85 breaths/min. The heart was exposed via left thoracotomy incision. The left coronary artery was identified and ligated proximally using a 7-0 polypropylene suture. Regional myocardial ischemia was confirmed by the  
25 rapid occurrence of akinesia in the area at risk. The wound was then closed.

### Isolation and Culture of Marrow-derived Stromal Cells

Isolation and primary culture of MSCs from the femoral and tibial bones of donor rats were performed  
30 according to Caplan's method (Wakitani S, Saito T, Caplan A.I. Myogenic cells derived from rat bone marrow

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mesenchymal stem cells exposed to 5-azacytidine. Muscle & Nerve 1995;18:1417-1426). After overdose with pentobarbital (100 mg/Kg given intraperitoneally), the femoral and tibial bones were collected. Both ends of the bones were cut away from the diaphyses. The bone marrow plugs were hydrostatically expelled from the bones with complete medium. The marrow plugs were disaggregated and the dispersed cells were centrifuged and resuspended twice in complete medium. These cells in 10 mL of complete medium were then introduced into tissue culture dishes. Medium was completely replaced every 3 days and the non-adherent cells discarded. Each primary culture was replated twice (first and second passages) to three new plates when the cell density within colonies became 80% to 90% confluent. After the twice-passaged cells became nearly confluent, they were harvested and used for the coronary infusion experiments.

#### Medium

The cells were routinely cultured in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing selected lots of 10% fetal calf serum and antibiotics (100 U/mL penicillin G, 100µg/mL streptomycin and 0.25µg/mL amphotericin B; all obtained from Gibco laboratories) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### Marrow-derived Stromal Cell Labeling

P+E86 murine ectropic retrovirus-packaging cells, which are derived from NIH 3T3 mouse fibroblasts, were obtained from Dr. Denis Cournoyer (McGill University, Montreal, QC, Canada) (Mompalmer RL, Laliberte J, Eliopoulos N, Beausejour C, Cournoyer D. Transfection of murine fibroblast cells with human cytidine deaminase cDNA confers resistance to cytosine arabinoside. Anti-Cancer Drugs 1996;7:266-274). The GP+E86 cells were transfected

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with the purified plasmid DNA pMFG-LacZ in a 10:1 molar ratio using the standard calcium phosphate transfection kit (Pharmacia, Baie d'Urfe, Quebec, Canada). The LacZ gene encodes for the production of bacterial  $\beta$ -galactosidase.

5 These cells were plated at 25% confluence for 48 hours. The second passaged MSCs growth medium was replaced with the supernatant from the GP+E86 cells (containing the replication-defective retrovirus carrying the  $\beta$ -gal reporter gene) to transfect the MSCs overnight and then replaced.

10 with normal complete medium for the following day. After three times of transfection, MSCs were then collected (approximately  $2 \times 10^6$  cells for one infusion) and resuspended in 50 $\mu$ L of serum-free DMEM and stored on ice until infusion into the ascending aorta. Some culture

15 plates were selected for histochemical staining *in vitro* for  $\beta$ -galactosidase activity. The cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate buffered saline (PBS) in 4°C for 5 minutes. Staining for  $\beta$ -gal was accomplished at 37°C for 16 hr in a solution containing 1

20 mg/ml 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside (X-gal), 2% dimethylsulfoxide, 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 1 mM magnesium chloride, and 0.02% Nonidet P-40 in PBS, pH7.3.

#### Infusion of Marrow-derived Stromal Cells

25 Two weeks after the coronary ligation, the 12 recipient rats were prepared for infusion of MSCs. Anesthesia was induced and maintained as above. The rats were intubated and connected to the ventilator. The ascending aorta was exposed through upper median sternotomy

30 and looped after dissection. Under direct vision, transfected MSCs suspension was then infused into the



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briefly distally clamped ascending aorta (about 20 seconds). After the infusion, the puncture bleeding site over ascending aorta was controlled by compression with gauze. The wound was then closed in layers.

5      Histology and Histochemical Staining for  $\beta$ -galactosidase Activity

Two rats were sacrificed immediately after the infusion. The hearts were excised and sliced along short axis of left ventricle to 3 mm thick sections in series,  
10 and fixed in 2% paraformaldehyde in PBS for 2 hours. The sections were then cryoembedded after protection with 20% sucrose in PBS overnight. The other 10 rats were taken for their final experiments 4 weeks after the MSCs infusion. After overdose with pentobarbital, the hearts were exposed  
15 and injected with 100 ml saline (0.9%) through the apex of the left ventricle, then perfusion-fixed with 2% paraformaldehyde in phosphate buffered saline (PBS). The hearts were excised, sliced and prepared as mentioned above. Cryosections 6 $\mu$ m in thickness were collected in each  
20 3 mm section sample across a set of gelatin-coated glass slides. One of every 10 cryosections was collected for histochemical staining for  $\beta$ -galactosidase activity as mentioned above. The sections were then counter-stained with hematoxylin and eosin. Tissue sections were examined  
25 with an Olympus microscope (BX-FLA, Olympus). Digital images, transferred to a computer equipped with Image Pro software (Media Cybernetics, MA), were subsequently printed.

Histochemical Stain for Gap-junction Protein, Connexin 43

30      The gap junctions which constitute cardiac muscle specific intercalated discs were demonstrated by histochemical stain for connexin 43, using rabbit anti-

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connexin antibodies (Zymed Laboratories Inc., San Francisco). Diaminobenzidine was used as a chromogen to produce the brown colour which represent gap junctions linking cardiomyocytes together (Nagy JI, Li WE, Roy C, Doble BW, Gilchrist JS, Kardami E, Hertzberg EL. Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin 43. Exp. Cell Res. 1997; 236:127 - 136).

### Results

10 Cultured MSCs were observed by phase microscope to assess the level of expansion and to verify the morphology at each culture medium change. Most of the hematopoietic stem cells were not adherent to the culture plate and removed with changes in medium. The adherent cells were  
15 seen as individual cells or colonies of only a few cells on day 6; however, they replicate rapidly and form colonies of up to 100 cells after the first week of culture. By the end of second week, the colonies of adherent cells have expanded in size, with each colony containing several  
20 hundred to several thousand cells. Adherent MSCs from rat legs have similar morphology, most being fibroblastic in appearance, with a few adipocytic, polygonal cells (Fig. 4). This phenotype was retained throughout repeated passages under nonstimulating condition.

25 We transfected twice-passaged MSCs with replication-defective retrovirus carrying the  $\beta$ -gal reporter gene as cell labeling before their implantation. The transfected MSCs showed clear histochemical staining for  $\beta$ -galactosidase activity (Fig. 5). Transfection efficiency of the MSCs  
30 culture was almost 100%.

The rats were sacrificed at the following intervals after the MSCs infusion: immediately for 2 rats; and 4

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weeks for 10 rats. Gross examination of the excised hearts shows clear myocardial scar formation (about 40 % of LV free wall) on all 12 recipient rats. Cryosections of the specimens were selected (as detailed in Methods) for

5 histochemical staining of  $\beta$ -galactosidase activity to trace and evaluate the morphology and phenotype changes of infused MSCs. Labeled cells could be identified in both rats sacrificed immediately after MSCs infusion, and in 6 out of 10 rats sacrificed 4 weeks later.

10 Immediately after MSCs infusion,  $\beta$ -gal positive cells were consistently found in all selected sections to be trapped within the coronary capillaries surrounded by endothelial cells all over the non-infarct area (Fig. 6). We failed to identify any  $\beta$ -gal positive cells in the

15 infarction zone at this time. Four weeks after MSCs infusion, some  $\beta$ -gal positive cells could be found within the normal myocardial area outside the infarction scar (Fig. 7). They have centrally located nuclei and are connected among themselves and with surrounding host

20 cardiomyocytes ( $\beta$ -gal negative cells) by intercalated disk-like structure, which are characteristics of normal cardiomyocytes. However, the  $\beta$ -gal positive cells also could be detected individually or in clusters within the myocardial scar (Fig. 8). They appear unorganized and

25 scattered in the infarction scar with fibroblast-like morphology, similar to that of the surrounding  $\beta$ -gal negative (host) fibroblast cells. Some  $\beta$ -gal positive cells were found incorporated into endocardium (Fig. 9) and coronary capillary endothelium within or outside the

30 infarct scar area (Figs. 10A & 10B).

Tissue sections showing labeled cells with histological features of cardiomyocytes (as in Fig. 7) were

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further studied immunohistochemically using antibodies against connexin 43, a major constituent protein of gap junctions in the intercalated discs of cardiac myofibers. The demonstration of such junctional structure (Fig. 11) further confirms the phenotype of the differentiated labeled cells, and their integration into the native cardiac myofibers.

#### INDUSTRIAL APPLICABILITY

According to the present invention, MSCs are determined to traffic through the circulatory system to the injured heart, and are capable of forming cardiomyocytes and other types of cells, depending on the specific microenvironment. In addition, endothelial progenitor cells in the MSCs population may be involved in the post-infarction neovascularization process. As a result, MSCs display myocardial cell differentiation properties *in vivo* and provide a promising therapeutic use in improving myocardial healing following infarction. Labeled cardiac myocytes and fibers were present in the implant site, which exhibited positive immunohistochemical stains in normal or immature cardiac myocytes. These findings indicate that the use of autologous marrow stroma cells (MSCs) in transplantation for myocardial regeneration in animals and humans.

In addition, the present invention has application in the area of experimental research, where by MSCs may be employed in a variety of *in vivo* animal models to further study the influence of the micro environments on stem cell differentiation, and on cellular signaling mechanisms.

Further echocardiographic studies are expected to demonstrate improved systolic thickening of the ischemic ventricular wall segment, and reduced ventricular size and

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remodeling, as had been reported following the implantation of other donor cells.

Future studies include clinical trials and mechanistic investigations employing the teachings of the present invention. Minimal medical and ethical difficulties are expected for clinical trials encompassing the present invention for the numerous reasons herein described. It is fully contemplated that the findings of the present invention will offer a valuable basis to pursue such scientific knowledge. For example, a comparison of the differentiation of labeled cells implanted at the center, and at the periphery of the infarcts will be made. In addition the micro-environments responsible for MSC differentiation in the myocardium will be further explored. Differentiation may be facilitated at the peripheral border zone, if direct cell-to-cell contact is an important signaling mechanism for such differentiation to take place. The role of cytokines and other growth factors will also be examined in the future. Further still, methodologies for transplanting autologous MSCs in patients to improve cardiac function will be optimized in future studies.

While the invention has been described in connection with specific embodiment thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses or adaptations of the invention following in general, the principles of the invention and including such departures from the present disclosure as come within the known customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.